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(54) Title: ISOCITRATE LYASE ENZYME FROM MYCOBACTERIUM TUBERCULOSIS AND INHIBITORY AGENTS TO COMBAT PERSISTENT INFECTION

(57) Abstract: The invention provides methods and compositions for use in identifying inhibitors of biochemical pathways important for persistent infection, allowing the identification and/or design of improved therapeutics for treating persistent infection by pathogenic microbes. Particularly disclosed is the importance of the glyoxylate shut to the persistent phase of various infectious agents, including Mycobacteria, such as *M.tuberculosis*, and the identification of preferred targets for drug development, including the enzymes isocitrate lyase (ICL) and malate synthase. Crystals and three-dimensional structures of *M.tuberculosis* ICL, without ligand and in complex with two inhibitors are also disclosed, for exemplary use in the design of inhibitors and therapeutic agents.

ISOCITRATE LYASE ENZYME FROM MYCOBACTERIUM TUBERCULOSIS AND INHIBITORY AGENTS TO COMBAT PERSISTENT INFECTION

BACKGROUND OF THE INVENTION

The present application claims priority to co-pending U.S. provisional patent application Serial No. 60/222,768, filed August 03, 2000, the entire text and figures of which application are specifically incorporated by reference herein without disclaimer. The U.S. Government owns rights in the present invention pursuant to Grant Numbers AI 43268 and GM 62410 from the National Institutes of Health.

1. Field of the Invention

The present invention generally relates to the fields of pathogenic microbes and to therapeutic agents for treating persistent infections, including infection by *M. tuberculosis*. Through rigorous definition of an important pathway for persistent infection, the invention provides preferred targets for drug development from the glyoxylate shunt pathway, such as the isocitrate lyase and malate synthase enzymes. Exemplary embodiments of the invention concern crystals and three-dimensional structures of *M. tuberculosis* isocitrate lyase in complex with inhibitors, for particular use in the design of inhibitors and therapeutic agents.

2. Description of Related Art

Although modern medicine has provided many weapons to combat disease, infection by pathogenic microbes still poses a significant threat to human life. In recent times, an increasing number of microbes have developed resistance to many commonly used antimicrobial agents, thereby contributing to a new spread of disease. Mycobacteria are examples of microbial pathogens that exhibit persistent infection. *Mycobacterium tuberculosis*, the causative agent of the tuberculosis (TB) disease, exhibits a penetrance in the human population that is rivaled by few other pathogens. Tuberculosis remains the largest cause of death in the world from a single infectious disease and causes many fatalities in developing countries.

The success of *M. tuberculosis* is dependent on its ability to persist and maintain chronic infection in humans (Parrish *et al.*, 1998). During chronic tuberculosis, the bacteria

exist in diverse metabolic states that are not targeted by conventional antimycobacterials (Mitchison, 1980). Lengthy regimens of anti-TB drugs are necessary and are currently the only way to even approach killing of the persistent bacteria.

The current drugs have further drawbacks, such as targeting only a small number of bacterial processes, notably cell wall formation and chromosomal replication (Parrish *et al.*, 1998; McKinney *et al.* 1998). The effectiveness of drugs aimed at intervening in such processes is further limited by the ability of the organisms to adapt under the selective pressure of the treatment and become resistant.

The emergence of drug resistant strains is a constant threat to the use of the currently available antimycobacterial agents. The development of multiple drug resistant strains of *M. tuberculosis* has resulted in fatal outbreaks of disease, including those in the United States. In fact, resistance to isoniazid, one of the most widely used anti-tuberculosis drugs for both therapy and prophylaxis, is now quite common. In recent years, resistance to isoniazid has been as high as 26% in some areas of the United States.

Despite ongoing efforts in this field, new drugs are thus urgently needed for use against TB and other microbes involved in persistent infection. As these pathogens are often able to evade currently available antimycobacterials, there is a particular need to identify better targets for drug development, leading to the identification or design of long sought after therapeutics. The development of agents particularly attuned to combating the persistent stage of infection would represent a significant advance in the art.

SUMMARY OF THE INVENTION

The present invention satisfies these needs in the art by providing important targets for use in the development of improved antimicrobials, particularly in the discovery and/or design of therapeutic agents to counteract the persistent stage of infectious pathogens. The invention is based, in part, upon the rigorous definition of pathways important for persistent infection, particularly the glyoxylate shunt pathway, and on the identification of the isocitrate lyase (ICL) and malate synthase enzymes as important targets for inhibition. In certain embodiments, the invention particularly provides crystallographic and three-dimensional

structural information for *M. tuberculosis* isocitrate lyase, without ligand and in complex with two inhibitors, for exemplary use in the design of inhibitors and therapeutic agents.

As used throughout the entire application, the terms "a" and "an" are used in the sense that they mean "at least one", "at least a first", "one or more" or "a plurality" of the referenced components or steps, except in instances wherein an upper limit is thereafter specifically stated. Therefore, an "inhibitor", for example, as used herein, means "at least a first inhibitor". The operable limits and parameters of combinations, as with the amounts of any single agent, will be known to those of ordinary skill in the art in light of the present disclosure.

In overall aspects, the present invention provides methods for identifying agents for use in treating or preventing persistent microbial infections. Such methods generally comprise first choosing or selecting an enzyme or "enzyme target" from, or closely functionally associated with, the glyoxylate shunt pathway of a pathogenic microbe that exhibits a persistent stage of infection. Next, the methods generally comprise identifying, selecting or designing a compound or agent that inhibits the chosen or selected enzyme target, thereby identifying and enabling the production of a compound, inhibitor or "agent" for use in treating or preventing a persistent microbial infection.

Currently preferred selected enzyme targets are isocitrate lyase and malate synthase, unique enzymes of the glyoxylate shunt. Other suitable selected enzyme targets are the malate dehydrogenase, citrate synthase and aconitase isoenzymes of the glyoxylate shunt. Further candidates as selected enzyme targets are acetyl CoA synthase, fructose-1,6-bisphosphatase and other acetyltransferases and transporters functionally related to the effective operation of the glyoxylate shunt.

These methods may utilize selected enzyme targets from or associated with the glyoxylate shunt pathway in any one of a variety of microbial pathogens, particularly intracellular microbial pathogens, such as those that cause persistent infections, most particularly intracellular microbial pathogens that participate in persistent infections in inflammatory macrophages *in vivo*.

Irrespective of the source of the selected enzyme targets and/or particular pathogen employed in the screening methods, due to the unifying nature of the glyoxylate shunt pathway across the spectrum of intracellular microbial pathogens, the compounds, inhibitors and agents identified by these methods will have broad spectrum activity across this class of microbes. For example, although a mycobacterial isolated enzyme or survival assay may be chosen, the compounds, inhibitors and agents identified will not be limited to uses against mycobacteria, but may be used against any pathogen target of the invention, such as fungi. However, should the intent be to particularly develop agents for use against a given pathogen, *e.g., M. tuberculosis,* it is evidently an advantage of the invention that selected enzyme targets from *M. tuberculosis* may be employed in these methods.

Thus, the selected enzyme targets may be enzymes isolated from or functional within mycobacteria, such as *M. tuberculosis or M. avium*; from pathogenic fungi, such as *C. albicans*; and from other organisms, such as *Pseudomonas, Salmonella, Yersinia,* and *Leishmania,* each of which cause persistent infection in animals and humans.

Aside from the source of the selected enzyme target or original microbial pathogen used in the screening methods, the compounds, inhibitors and agents so identified may inhibit the same or the counterpart selected enzyme target from a mycobacterium, thereby identifying an agent for use in treating or preventing a persistent mycobacterial infection. Wherein, the compounds, inhibitors and agents so identified inhibit the same or the counterpart selected enzyme target from *M. tuberculosis,* such compounds, inhibitors and agents are effective for use in treating or preventing persistent or chronic tuberculosis. Irrespective of the source of the selected enzyme target or original microbial pathogen, the compounds, inhibitors and agents so identified may inhibit the same or the counterpart selected enzyme target from a fungus, thereby identifying an agent for use in treating or preventing a persistent fungal infection.

One of the advantageous insights of the present invention is that the selected enzyme target, whether isolated or obtained from, or maintained present within the intact host, should be a selected enzyme target from an intracellular microbial pathogen grown on a carbon source *in vitro* that mimics the nutrient environment encountered during the persistent phase of infection *in vivo*. Accordingly, the screening or other means of inhibitor identification will

facilitate the identification of compounds, inhibitors and agents will therapeutic or prophylactic utility. In particular, the methods will favor the identification of compounds, inhibitors and agents that are effective against treating or preventing the persistent phase of infection, thus satisfying the most urgent need in the art.

Although preferably obtained from a microbial pathogen grown on a carbon source *in vitro* that mimics the nutrient environment encountered during the persistent phase of infection *in vivo*, compounds that inhibit the selected enzyme target may be either pre-selected or identified by testing the ability of candidate compounds to inhibit the activity of the selected enzyme target in a cell-free enzyme activity assay.

In other methods, compounds that inhibit the selected enzyme target may be identified more directly by testing the ability of candidate compounds to inhibit the growth of the intracellular microbial pathogen when grown on a carbon source *in vitro* that mimics the nutrient environment encountered during the persistent phase of infection *in vivo*. When such methods are used, the candidate compound may have been pre-screened using the foregoing type of isolated enzyme assay.

The "growth on a carbon source *in vitro* that mimics the nutrient environment encountered during the persistent phase of infection *in vivo*" means that the rich media, typically employed without concern in the prior art, should be avoided. Exemplary carbon sources for use *in vitro* that mimic the nutrient environment of the persistent phase *in vivo* infection are C_2 carbon sources, such as acetate.

Other preferred embodiments of this invention are those aspects that concern identifying compounds and inhibitors by testing the ability of candidate compounds to differentially inhibit the growth of an intracellular microbial pathogen when grown *in vitro* on two different carbon sources, a first carbon source that mimics the nutrient environment encountered during the persistent phase of infection *in vivo*, *i.e.*, that induces the glyoxylate shunt, and a second carbon source that renders the glyoxylate shunt dispensable. For example, the first carbon source will be a C_2 carbon source, such as acetate, and the second carbon source will be a C_6 carbon source, such as glucose.

Under such conditions, the preferred candidate inhibitors are those that exhibit "differential inhibitory properties". That is, those that have the ability to preferentially inhibit microbial growth or survival on a C_2 carbon source in comparison to growth or survival on a C_6 carbon source. Preferably, the chosen candidate inhibitors will significantly inhibit microbial growth or survival on a C_2 carbon source, such as acetate; and will not significantly inhibit microbial growth or survival on a C_6 carbon source, such as glucose.

In certain other preferred embodiments, compounds that inhibit the selected enzyme target(s) are identified by at least a two-part screening technique. In such methods, the candidate compounds are first tested for the fundamental ability to inhibit the activity of the selected enzyme target in a cell-free enzyme activity assay. Positive candidate inhibitors from the enzyme assays are then tested for the ability to inhibit the selected enzyme target in intact cells, preferably when the microbes are grown on a carbon source *in vitro* that mimics the nutrient environment encountered during the persistent phase of infection *in vivo*.

A particularly useful practical embodiment of this inventive technique is a screening method that comprises:

(a) testing the ability of a candidate compound to inhibit the activity of a selected enzyme target in a cell-free enzyme activity assay, thereby selecting a positive candidate inhibitor; and

(b) further testing or confirming the ability of the positive candidate inhibitors from the cell-free enzyme activity assay to significantly inhibit the growth of the intracellular microbial pathogen when grown on acetate *in vitro*, but to not significantly inhibit the growth of the intracellular microbial pathogen when grown under appropriately controlled conditions, but on glucose, thereby favoring the selection of an inhibitor that preferentially inhibits an enzyme of the glyoxylate shunt pathway operative in persistent infection of inflammatory macrophages *in vivo*.

The invention further provides the motivation to prepare or "grow" crystals of the selected enzyme targets so that their crystal structure can be determined and candidate

inhibitors selected or designed from an understanding of such three-dimensional structural information.

Accordingly, the invention provides screening methods wherein a compound that inhibits the selected enzyme target is identified by a method comprising:

(a) preparing a crystal of the selected enzyme target;

(b) obtaining the atomic coordinates of the selected enzyme target by X-ray diffraction studies using the crystal;

(c) using the atomic coordinates to define the catalytic active site of the selected enzyme target; and

(d) identifying or designing a non-native substrate compound that fits the catalytic active site, thereby identifying a candidate compound that inhibits the selected enzyme target.

In exemplary embodiments, the invention provides methods for preparing isocitrate lyase and malate synthase crystals, and provides the resultant crystals and three-dimensional structural information. The crystals and three-dimensional structural information are preferably for microbial enzymes. In certain embodiments, the sources of the enzymes are mycobacterial, such as from *M. tuberculosis*, and in other embodiments, the sources of the enzymes are fungal, such as from *C. albicans*.

The crystals and three-dimensional structural information are also preferably for enzymes that include an ordered active site. As such, the invention particularly provides a crystallized mycobacterial, or *M. tuberculosis*, isocitrate lyase enzyme that includes an ordered active site. In certain embodiments, the invention provides a crystallized microbial isocitrate lyase enzyme other than an isocitrate lyase enzyme from *E. coli* and other than an isocitrate lyase enzyme from *Aspergillus nidulans*.

In certain preferred embodiments, the invention provides a crystallized *M. tuberculosis* isocitrate lyase enzyme without ligand, *i.e.*, apo-ICL, that has the crystallographic data of Table 1. Crystallized apo-ICL that has the crystallographic or atomic coordinates of FIG. 5, as deposited in the Protein Data Bank under accession code 1F61 is another embodiment of the invention.

Further aspects of the invention are the structure of ICL of FIG. 2, and a computer-readable data storage medium comprising a data storage material encoded with computer-readable data, wherein the data comprises the structural information for ICL as represented in FIG. 2.

Additional embodiments of the invention are an ICL tetramer wherein each subunit is composed of 14 α-helices and 14 β-strands; wherein the eight α-helices (α4-α11) and eight β-strands (β2-β5, β8, β12-β14) of the largest domain form an α/β-barrel that has a topology of $(\beta\alpha)_2\alpha(\beta\alpha)_5\beta$; wherein helix α12, present after the eighth β-strand, projects away from the barrel; wherein helices α12, α13 and α14 form interactions exclusively with the neighboring subunit; wherein residues 184-200 and 235-254 form a small β-domain of a short five-stranded β-sheet (β6, β7, β9, β10, β11) which lies atop the α/β-barrel; and a computer-readable data storage medium comprising a data storage material encoded with computer-readable data, wherein the data comprises the structural information for a so-defined ICL tetramer.

Certain preferred embodiments of the invention are ICL crystals and the three dimensional structure of such ICL crystals in combination with an inhibitor, such as 3-brompyruvate or 3-nitropropionate. In addition to native enzymes, mutant forms of ICL may be used throughout and may prove advantageous in certain embodiments, as shown by the data herein.

Exemplary methods for making crystals of microbial, mycobacterial or *M. tuberculosis* isocitrate lyase enzymes are provided that comprise:

(a) crystallizing a microbial, mycobacterial or *M. tuberculosis* isocitrate lyase enzyme by vapor diffusion using a buffer comprising about 100 mM HEPES and about 1.4 M sodium citrate at about pH 7.5; or

(b) incubating a microbial, mycobacterial or *M. tuberculosis* isocitrate lyase enzyme with glyoxylate, modifying the incubated enzyme with 3-bromopyruvate and crystallizing the modified enzyme in the presence of a buffer comprising about 0.1 M Tris-HCl, about 0.2 M sodium acetate, about 20-30% PEG4000 at about pH 8.0.

In terms of inhibitor complexes, the invention particularly provides crystallized ICL in complex with the inhibitor 3-brompyruvate that has the crystallographic or atomic coordinates of FIG. 6, as deposited in the Protein Data Bank under accession code 1F8M; and crystallized ICL in complex with the inhibitor 3-nitropropionate that has the crystallographic or atomic coordinates of FIG. 7, as deposited in the Protein Data Bank under accession code 1F8I.

The invention also provides the structures of the bound complexes of FIG. 3, and a computer-readable data storage medium comprising a data storage material encoded with computer-readable data, wherein the data comprises the structural information for the bound complexes of FIG. 3.

Further embodiments of the invention are thus structural determinants of a mutant ICL in which Cys 191 is changed to Ser and in which a crystal is formed with glyoxylate or 3-nitropropionate, such that the glyoxylate binds by coordination to the active site Mg^{2+} ion and forms hydrogen bonds with residues Ser 91 OG, Gly 92 N, Trp 93 N and Arg 228 NH2; structural determinants in which a succinate molecule is fit to the density such that one carboxylate makes specific hydrogen bonds with the side chains of residues Asn 313 ND1, Glu 295 OE2, Arg 228 NH1 and Gly 192 N, while the second carboxylate forms hydrogen bonds with Thr 347 OG, Asn 313 ND2, Ser 315 OG, Ser 317 OG and His 193 ND1, wherein the protein surface that packs against the C2 and C3 methylene carbons of succinate is provided by residues Trp 93, Thr 347 and Leu 348, and the C2 carbon of succinate is located